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In Re Application of

Maria Anna Wubben et al.

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For: PECTINS AS FOAM STABILIZERS FOR BEVERAGES HAVING A
FOAM HEAD



DECLARATION of Alexandra J.M. Wijsman

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Alexandra Johanna Mathilda Wijsman herewith declares as follows:

1. I presently reside at Hondsdraveld 23, 3448 EC Woerden, the Netherlands.

2. I am an employee of Heineken Technical Services B.V. at Zoeterwoude, the Netherlands, the assignee of the present invention. I am a graduate of the Agricultural University of Wageningen, with a specialization in food technology. Since 1995 I have been involved in research on raw materials for the production of beer, such as polysaccharides.

3. In order to determine the effect of the composition of a hop-pectin preparation on foam stability of beer, experiments were conducted where the amount of AUA in a hop pectin preparation obtained was increased and the amount of non-pectin components such as proteins was decreased by purifying the initial hop pectin preparation. Both the initial as well as the resulting hop pectin preparations were used for foam stability tests.

4. I conducted part of the experiments ; the other parts were conducted in my presence. The experiments and results are as follows.

Pectin preparation A (produced on industrial scale) was obtained from CO₂ extract residues by an extraction procedure using the same principles as described in the patent application on page 14, however, modified to take into account industrial size unit operations. The CO₂ extract residues were ground and mixed with warm acidified water. This suspension was filtrated to remove the solids. The extract obtained was mixed with alcohol to precipitate the pectin. The precipitate was separated from the solution and was washed out twice with alcohol after which the pectin was dried and hop pectin preparation A was obtained.

5. The purity of the isolated pectin preparation A was determined as percent anhydrogalacturonic acid (AUA) content (JEFCA: Compendium of food additive specifications). These galacturonic acid residues compose the backbone of pectin. The backbone is interrupted by many side chains containing a certain amount of neutral sugars. This structure of pectin is also described in Food colloids (p.420):

"The major structural feature of the pectic substances is the presence of acid polysaccharides composed largely of D-galacturonic acid units linked through α-(1-4) glycosidic bonds. Normally some of the carboxyl groups are esterified with methyl alcohol. The neutral sugar content is dominated by D-galactose, L-arabinose, and L-rhamnose with lesser amounts of D-xylose, L-fucose, 2-o-methyl-D-xylose and 2-o-methyl-L-fucose. Portions of the neutral sugars occur as neutral polysaccharides, arabans, galactans, and arabinogalactans. Some of these polysaccharides may be removed from pectic substances by redissolving the pectin in water and precipitating the pectin with alcohol. Part of the neutral polysaccharides tend to remain in solution, but removal of all the nonuronide residues by repeated precipitations is usually impossible."

6. The pectic polysaccharides are probably the most complex class of cell-wall polysaccharides and comprise a family of acidic polymers like homogalacturonans, rhamnogalacturonan and several neutral polymers like arabinans, galactans and arabinogalactans attached to it. Therefore, the amount of neutral sugars present in the hop pectin preparation was also determined by Gas Liquid Chromatography after hydrolysis with trifluoroacetic acid (H.N. Englyst and J.H. Cummings, *Analyst* 109 (1984) 937-942.) and conversion to alditol acetates. In addition, protein content was determined according to Kjeldahl (J.P. Roozen and L. van Boxtel, *De Ware(n) Chemicus*, 9 (1979) 192-195.), using a nitrogen content of 6.25%. The phenolic compounds were determined according to Folin-Cocaltieu (A. Scalbert, B. Monties, and G. Janin, *J. Agric. Food Chem.*, 37 (1989) 1324-1329.).

Tannic acid was used as a standard. The water content was determined after drying overnight at 105°C. The ash content was determined after pre-heating at 200°C during 1 h followed by heating at 550°C during 3 h. The phosphorus content was determined colorimetrically according to P.S. Chen, T.Y. Toribara, and H. Warner, *Analytical Chem.*, 28 (1956) 1756-1758.

7. The hop pectin preparation A was added to beer to determine the influence thereof on the foam stability. This experiment was repeated several times with different batches of reference pilsner beer. Hop pectin preparation A was dissolved in 5 ml water before being added to reference pilsner beer in an amount equivalent to 10 g pectin preparation per hectolitre of beer (30 mg pectin preparation / bottle of beer), which equals for this hop pectin preparation A an addition of 3.08 g AUA (anhydrogalacturonic acid) per hectolitre of beer. The bottles were gently shaken for 48 hours at room temperature. The foam stability was then determined using the Nibem foam meter.

8. In order to increase the purity of hop pectin preparation A, and hence to decrease the amount of non pectin components in this hop pectin preparation, a sub-sample of hop pectin preparation A was subjected to an extra acidic alcohol washing step. Subsequently the hop pectin preparation was washed with alcohol to remove the acid. After drying, a second hop pectin preparation B was obtained which originated from pectin preparation A and hence originated from the same starting material (the CO₂ extract residues). Similar to hop pectin preparation A, the purity (= anhydrogalacturonic acid) of this hop pectin preparation B was determined. In addition, neutral sugars, protein, polyphenols, water, ash and phosphorus content was also determined as described above. All these analyses of hop pectin preparation B were carried out simultaneously with the analyses of hop pectin preparation A.
In accordance with hop pectin preparation A, the influence of hop pectin preparation B on foam stability of beer was also determined as described above. Hop pectin preparation B was dissolved in 5 ml water before being added to reference pilsner beer in an amount of 10 g pectin preparation per hectolitre of beer (30 mg pectin preparation / bottle of beer), which equals for this hop pectin preparation A an addition of 4.29 g AUA (anhydrogalacturonic acid) per hectolitre of beer. This

experiment was also repeated several times with different batches of reference pilsner beer.

9. The results of the experiments were as follows:

The composition of hop pectin preparations A and B, as determined with the above described methods, is presented in table 1.

Table 1. Chemical composition of hop pectin preparations A and B.

Composition		Hoppectin A	Hoppectin B
Anhydrogalacturonic acid (AUA)	% w/w	30.8	42.9
Neutral sugars	% w/w	19.8	16.5
Protein (denatured)	% w/w	11.4	8
Phenolic compounds	% w/w	2.6	2.5
Ash	% w/w	19	12.9
Phosphorous	% w/w	1.4	0.5
Water	% w/w	1.4	6.5

86.4% 89.8%

The extra acid alcohol washing step used for hop pectin preparation B compared to hop pectin preparation A resulted in an increase in anhydrogalacturonic acid (AUA) content of the pectin preparation of 12 % w/w. Moreover, the non pectin components such as protein and ash were significantly reduced by respectively 3.4% w/w and 6.1% w/w.

10. The results of the foam stability experiments with pectin preparations A and B are presented in tables 2a, b and c and Figure 1. The values given in table 2a represent foam numbers (duplicate measurements) of reference beer, beer with 10 g / hl added hop pectin preparations A and B (addition equivalent to respectively 3.08 and 4.29 g AUA / hl beer), and foam improvement established by respectively hop pectin preparation A and B (calculated as average foam number pectin beer minus average foam number reference beer). The overall average foam improvement values of hop pectin preparations A and B are also graphically presented in Figure 1. The given foam data are derived from 7 individual experiments using different batches of reference beer, each performed in duplicate. In each of

these experiments both hop pectin preparation A as well as hop pectin preparation B were tested together with the same reference pilsner beer.

11. In addition, an extra experiment was conducted in which hop pectin preparations A and B were both added to beer in a similar amount of 5 g AUA / hl. Foam stability was measured together with a reference pilsner beer. These foam data are presented in table 2b.

12. Statistical analysis of the data was performed with Analyse-It, an add-in of Excel. ANOVA (Analysis of Variance, a statistical technique) can be used when we have multiple samples, for which we want to know if they have the same average. Or, stated more precisely: with ANOVA it can be calculated what the probability is that all data is drawn from the same population, i.e. have the same means. ANOVA is used to make a statement about the means of the three samples, the reference, hop pectin preparation A and hop pectin preparation B, to establish whether their foam number can be considered similar or not.
In addition, foam improvements of hop pectin preparations A and B are compared using a t-test. The t-test used is a statistical method to determine whether two samples are likely to have come from one underlying population, thus with the same means. In practical terms this means that with a t-test, we can test whether two averages are to be considered statistically equal or whether there is a difference between the two averages. The statistical evaluation of the data presented in table 2a is given in table 2c.

Table 2a. Comparison of foam number and corresponding foam improvement of hop pectin preparation A containing 30.8% w/w AUA and hop pectin preparation B containing 42.9% w/w AUA, both added in an amount of 10 g pectin preparation / hl

Experiment	Foam number (sec)			Foam improvement (sec)	
	reference	hop pectin A	hop pectin B	hop pectin A	hop pectin B
1	257	288	349	34	89
	249	286	335		
2	252	290	332	37	82
	253	291	337		
3	237	288	307	50	82
	239	288	332		
4	276	321	363	37	86
	280	308	364		
5	275	323	353	47	79
	283	329	362		
6	267	303	345	34	81
	282	314	365		
7	281	340	400	54	121
	275	323	397		
average	265	307	353	42	89
stdev	16.4	18.4	25.2	8.3	14.7

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Table 2b. Comparison of foam number and corresponding foam improvement of hop pectin preparation A containing 30.8% w/w AUA and hop pectin preparation B containing 42.9% w/w AUA, both added in an amount of 5 g AUA / hl beer.

Samples	Pectin addition g pectin preparation/hl	Pectin addition as AUA g AUA / hl	Foam number (sec)			Foam improvement (sec)
					average	
Reference	-	-	267	282	275	-
Hop pectin A	16.3	5	311	321	316	42
Hop pectin B	11.7	5	332	359	346	71

Table 2c. Statistical evaluation of the data presented in table 2a.

ANOVA

H_0 : no difference in foam number between reference, hop pectin A and hop pectin B

All variation is random

All groupmeans are equal to the overall mean

H_1 : foam numbers of reference, hop pectin A and hop pectin B are different

$\alpha = 0.05$

1-way between subjects ANOVA analysed with: Analyse-It + General v1.44

Foam number (sec): reference, hop pectin A, hop pectin B

foam number (sec)	n	Mean	SD	SE
reference	14	264.714	16.434	4.3921
hop pectin A	14	306.571	18.425	4.9244
hop pectin B	14	352.929	25.190	6.7323
Source of variation				
foam number (sec)	SSq	DF	MSq	F
	54519.571	2	27259.786	65.73
Within cells	16173.214	39	414.698	
Total	70692.786	41		<0.0001

p-value < 0.0001 (probability that H_0 is correct)

p-value < 0.05 (α): H_0 is too unlikely to be acceptable, so we reject H_0 and accept that the foam numbers of the reference, hop pectin A and hop pectin B are different.

Independent samples t-test:

H_0 : foam improvement of hop pectin A is equal to that of hop pectin B.

the observed difference between the two averages is solely based on randomness

$\mu_A = \mu_B$

H_1 : foam improvement of hop pectin A and B are different

the observed difference is not based on a random process but on a structural difference between the two averages.

$\mu_A \neq \mu_B$

$\alpha=0.05$

Independent samples t-test analysed with: Analyse-It + General v1.44

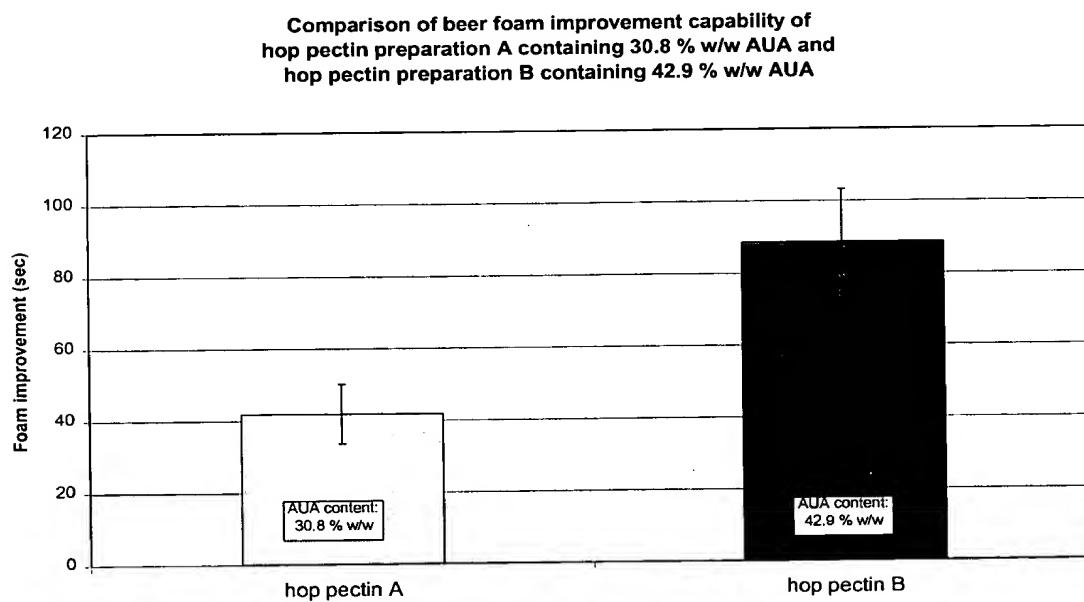
Foam improvement (sec): H_0 : hop pectin A = hop pectin B

foam improvement (sec)	n	Mean	SD	SE
hop pectin A	7	41.857	8.275	3.1277
hop pectin B	7	88.571	14.684	5.5500
Difference between means	-46.714			
95% CI	-60.595 to -32.834			
t statistic	-7.33			
2-tailed p	<0.0001			

2-tailed p < 0.0001 (probability that H_0 is correct)

2-tailed p < 0.05 (α): H_0 will be rejected. With an accuracy of 95% we can say that foam improvement of hop pectin preparation B is different from hop pectin preparation A.

Figure 1. Average foam improvement capability of hop pectin preparations A and B, both added in an amount of 10 g pectin preparation per hectolitre of beer



13. In all experiments which were conducted, using several batches of reference pilsner beer and the same dosing of both hop pectin preparations A and B equivalent to 10 g pectin preparation / hl beer, pectin preparation B had a statistically significant higher foam improvement in seconds than pectin preparation A. Due to the additional purification step applied, hop pectin preparation B had a higher anhydrogalacturonic acid content and less non pectin components than hop pectin preparation A (see table 1). Since the concentration of mainly ash and protein were markedly decreased resulting in a relative increase in anhydrogalacturonic acid (AUA) content of 39.3% it can be concluded that the non pectin components such as ash and protein do not contribute to the observed foam positive effect of the hop pectin preparation B, showing an average foam improvement 112% higher than hop pectin preparation A. The test data show that as the amount of anhydrogalacturonic acid in the hop pectin preparation increases, the foam stability also increases. Moreover, it could be argued that the above mentioned non pectin constituents have a foam negative effect since the foam improvement of hop pectin preparation B added in an amount equivalent to 5 g AUA / hl is 69% higher compared to the foam improvement of hop pectin preparation A added in a similar amount (table 2b). These results show that the pectin component is the component responsible for the foam stability and not the non pectin components present in the hop pectin preparation.

14. In my Declaration dated 13 July 1999 I have reported experiments comparing the effect of hop-pectin preparations on foam stability with the effect of beet pectin preparations on the foam stability of beer.

One commercial beet pectin preparation, in said Declaration designated as beet pectin 2, was analysed to determine the content of anhydrogalacturonic acid (AUA) (JEFCA: Compendium of food additive specifications) and the amount of neutral sugars (Gas Liquid Chromatography after hydrolysis with trifluoroacetic acid (H.N. Englyst and J.H. Cummings, *Analyst* 109 (1984) 937-942.) and conversion to alditol acetates). In addition, protein content was determined according to Kjeldahl (J.P. Roozen and L. van Boxtel, *De Ware(n) Chemicus*, 9 (1979) 192-195.), using a nitrogen content of 6.25%. The phenolic compounds were determined according to Folin-Cocaltieu (A. Scalbert, B. Monties, and G. Janin, *J. Agric. Food Chem.*, 37 (1989) 1324-1329.). The amount of Tannic acid was used as a standard. The ash content was determined after pre-heating at 200°C during 1 h followed by heating at 550°C during 3 h. Warner, *Analytical Chem.*, 28 (1956) 1756-1758.

The composition of the beet pectin preparation was as follows:

Composition	Beet pectin 2	
Anhydrogalacturonic acid (AUA)	% w/w	59.9
Neutral sugars	% w/w	19.7
Protein (denatured)	% w/w	4.3
Phenolic compounds	% w/w	1.4
Ash	% w/w	2.6

27.7

On the basis of my experience, training and expertise in the area of improving foam stability with the addition of pectin preparations and also comparing this beet pectin preparation composition with the hop pectin preparation compositions described in Paragraph 9 above, it is my opinion that the non-pectin components in the hop pectin preparations and in the beet pectin preparation perform in substantially the same way with regard to foam stability.

15. The undersigned declares that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that wilful false statements so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements so made may jeopardize the validity of the document, or application, or any patent issuing thereon.

Signed this 15th day of August, 2000

By 
Alexandra J.M. Wijsman